Sex- and tissue-specific, but hormonally independent, demethylation at the 3′-end of *Xenopus* vitellogenin gene B1

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We describe three *Hpa*II-specifying sequences in 6 kbp of DNA at the 3′-end of *Xenopus* vitellogenin gene B1 (two within the coding sequence and one downstream) which remain methylated in all tissues of adult male *Xenopus*. These sites are however fully demethylated exclusively in adult female *Xenopus* liver parenchymal cells but not in oviduct, kidney or erythrocytes. Developmental studies suggest that this tissue- and sex-specific demethylation may occur gradually after metamorphosis. The methylation of these 3′-sites is independent of transcriptional activation by oestrogen of vitellogenin genes in male or female *Xenopus*.

DNA methylation *Vitellogenin gene expression* *Estrogen* *Sex-specificity* *Tissue-specificity*

1. INTRODUCTION

It has often been suggested that the loss of methyl groups at the 5′-position of cytosine in CpG residues of DNA sequences is associated with, but not solely sufficient for, the activation of specific gene transcription [1–3]. The differential specificities of the restriction enzymes *Hpa*II and *Msp*I in cleaving the sequence CCGG, according to whether or not the internal cytosine is methylated, has greatly facilitated the analysis of a correlation between DNA methylation in and around a given gene and its transcriptional activity [2,4,5]. We have been interested in establishing the structural and developmental basis of the reversible activation by oestrogen of the vitellogenin multigene family in male and female *Xenopus* hepatocytes [6–9]. Recent studies from other laboratories have suggested that, unlike most developmentally and hormonally activated genes [3], the 5′-flanking and coding sequences of the *Xenopus* vitellogenin genes A1 and A2 remained fully methylated during their active transcription [10,11], while an *Hpa*II site at the 5′-end of chicken vitellogenin gene II undergoes demethylation only after oestrogen-induced transcription [12]. However, no information is available on possible demethylation occurring at the 3′-end of these genes.

The *Xenopus* vitellogenin gene family comprises 4 expressed genes termed A1, B1, A2 and B2 [13]. The A1 and B1 genes are linked as 5′–A1–B1–3′ with a 15 kb of DNA spacer [14]. This gene pair is more actively transcribed in both male and female *Xenopus* hepatocytes upon induction with oestrogen than genes A2 and B2, while gene B1 is transcriptionally the most active gene at early stages of induction [9]. An important characteristic of vitellogenin genes in vertebrates is that the normally dormant genes in the male can be rapidly activated de novo in the liver by the administration of oestrogen [15,16]. In *Xenopus* hepatocytes, all 4 vitellogenin genes are thus transcribed at the same rate and to the same extent as in females [7,9]. It was therefore interesting to test whether hormonal activation of vitellogenin genes would be paralleled by a tissue-specific demethylation at the 3′-end of the relatively most actively transcribed B1 gene.

2. MATERIALS AND METHODS

Adult and immature *Xenopus* were obtained

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from Xenopus Ltd (Redhill, England) and maintained at 21°C. Where indicated animals were injected with 2 mg (adults) or 1 mg (immature animals) of oestradiol-17β dissolved in propane-1,2-diol. For long-term treatment, cholesterol pellets containing 12 mg (adults) or 2–3 mg (immature animals) of oestradiol were implanted subcutaneously. The animals were chilled in ice and various tissues removed rapidly for extraction of DNA. In the case of liver tissue, parenchymal cells in which vitellogenin genes alone are expressed [17] were enriched to 95% before DNA was extracted. The enrichment was accomplished by separation of liver derived cells by Percoll density centrifugation [7]. DNA was isolated from tissue samples pooled from 6–8 adult (>4 year old) animals [18]. Aliquots of 7–10 μg DNA were digested with a 10–30-fold excess of restriction enzyme by overnight incubation at 37°C. It was verified that both the *Hpa*II + *Eco*RI and *Msp*I + *Eco*RI double digestions gave identical patterns with the B1 vitellogenin genomic clone λX13, while completeness of digestion was monitored by the parallel digestion of λDNA in a 20% aliquot of the main digestion mixture. Digested DNA was extracted with phenol/chloroform before it was electrophoretically resolved on 0.8% agarose gel for approx. 24 h at 1.5 V/cm. DNA was then transferred onto Schleicher and Schuell nitrocellulose filters [19] and prehybridized overnight at 42°C in 50% formamide, 5 × SSC (1 × SSC = 150 mM NaCl, 15 mM trisodium citrate), 0.1% SDS, 10 × Denhardt's solution (1 × Denhardt's solution = 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone), 100 μg/ml sonicated denatured calf thymus DNA, 100 μg/ml poly(A). Hybridization was carried out overnight at 37°C in the above solution (except that 5 × Denhardt's solution was used) and with the addition of 10% dextran sulphate, 10 μg/ml sonicated, denatured *Xenopus laevis* DNA and denatured ^{32}P-nick-translated λX13 as the probe.

Filters were washed twice at 42°C for 2 h in 2 × SSC, 0.1% SDS followed by two washings for 1 h each in 0.2 × SSC, 0.1% SDS, 68°C and finally for 10 min in 0.2 × SSC at room temperature before they were exposed to Fujimax X-ray film at −70°C in the presence of two Dupont intensifying screens for 24–96 h.

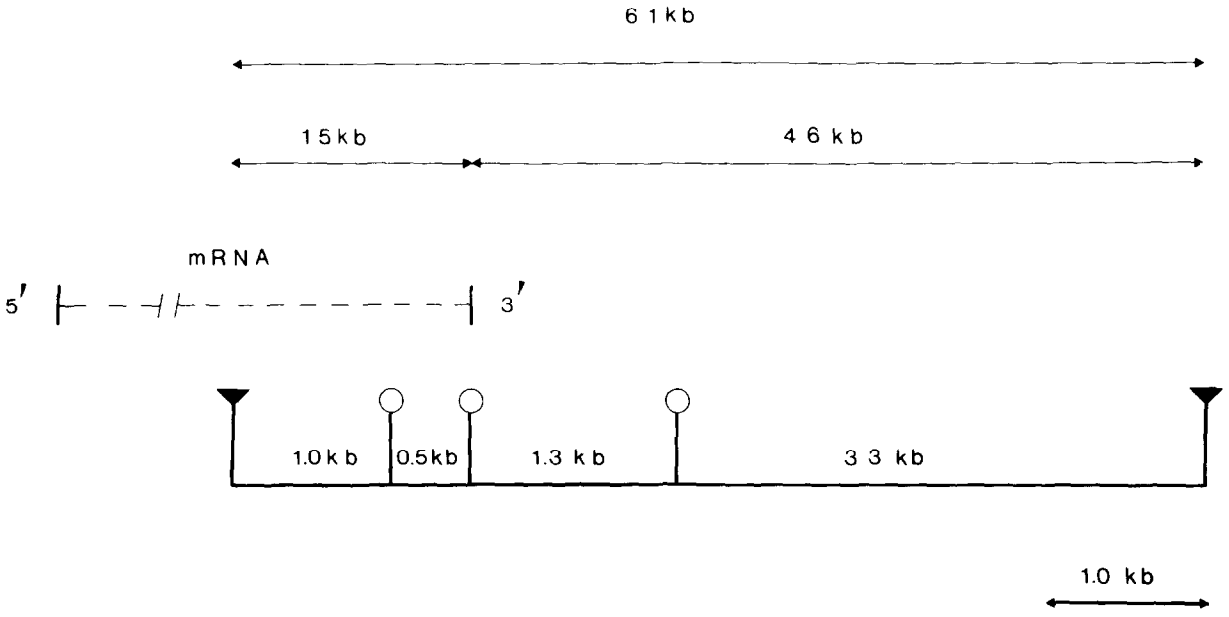

Fig.1. Simplified map of clone λX13 specifying the 3'-end of the B1 vitellogenin gene of *Xenopus laevis*. The clone λX13 was bordered by *Eco*RI sites (▼) with 3 internal *Msp*I/*Hpa*II sites (○). Note that both the B1 vitellogenin mRNA and the cloned 3'-genomic DNA of this gene are about 6 × 10^3 nucleotides in size.

3. RESULTS AND DISCUSSION

Fig.1 depicts a simple restriction map of the 6.1 kbp cloned DNA at the 3′-end of vitellogenin gene B1 that we have derived from an *Eco*RI genomic clone bank in λ Charon 4 phage (clone λX13). It also shows the sizes of the major products that would be generated by double digestion with *Eco*RI and *Msp*II or *Hpa*II restriction enzymes. Two of the three CCGG methylation sites are located within 500 bp of the 3′-end of coding sequences while another is approx. 1.3 kbp downstream. DNA from different tissues of adult (>4 years) male and female *Xenopus* was cleaved with a combination of restriction enzymes *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I or *Eco*RI alone and, after electrophoresis, hybridized with ^{32}P-labelled λX13 as the probe. The results shown in fig.2 demonstrate that, except for female liver, DNA from male liver and all other male and female tissues was fully methylated at the 3′-sites, as seen from the absence of cleavage with *Hpa*II. Even in the oviduct which is a major target for oestrogen, these sites in the vitellogenin gene B1 remain methylated. This shows that their demethylation in female liver is both tissue- and sex-specific. The occasional and faint appearance of low-M_r bands in the 'negative' lanes (i.e., for DNA from oviduct or male kidney) may reflect cutting in the DNA of a small proportion of cells in these tissues.

Fig.3 shows that 3 days after a saturating dose of oestradiol, when all 4 genes would be transcribed at their maximum rate [9], these sites were not demethylated in the male. Whether or not exogenous hormone was administered, this gene in female hepatocytes was fully demethylated. Fig.3 also shows that a slow but long-lasting demethylation produced as a consequence of transcriptional activation as suggested for other genes [12,20,21], was unlikely when the DNA was analyzed 40 days

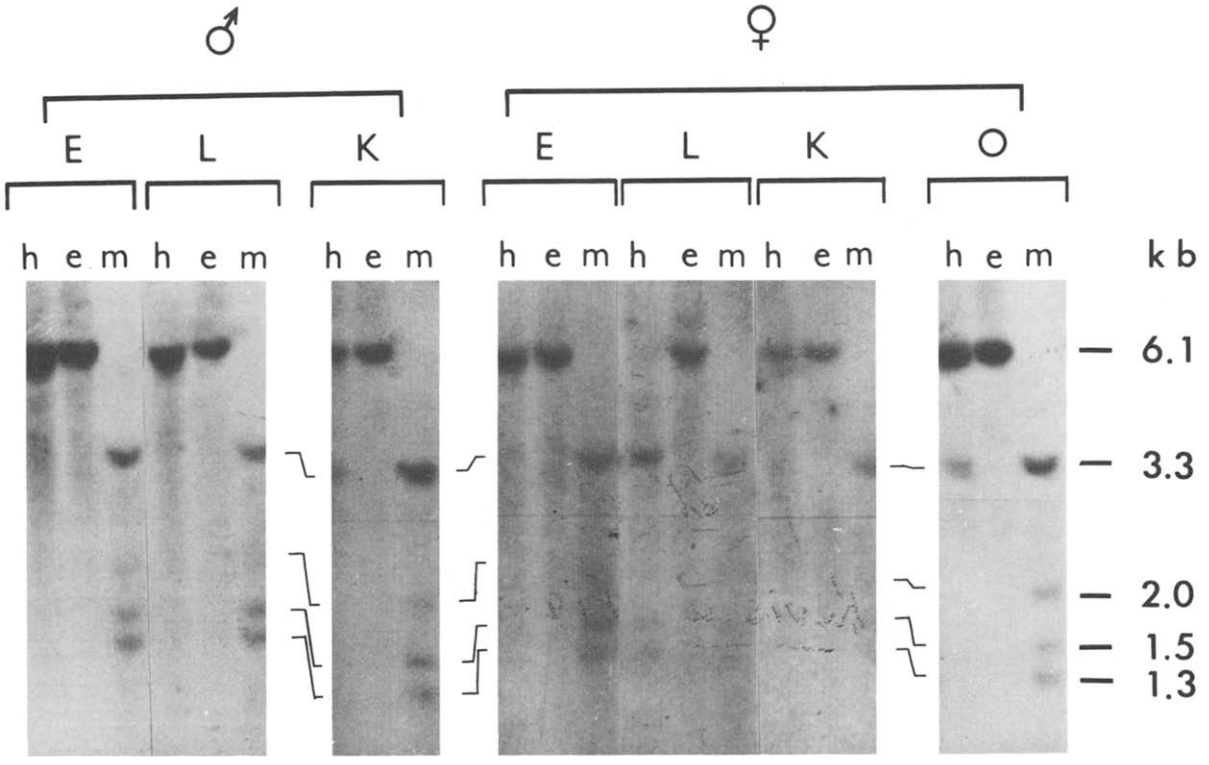

Fig.2. Methylation pattern, as judged by differential digestion with restriction enzymes *Hpa*II and *Msp*I of *Xenopus* vitellogenin gene B1 in DNA from different tissues of adult male and female *Xenopus*. Tissues: E, erythrocytes; L, liver; K, kidney; O, oviduct. Restriction enzyme digestion: h, *Eco*RI and *Hpa*II; e, *Eco*RI alone; m, *Eco*RI and *Msp*I.

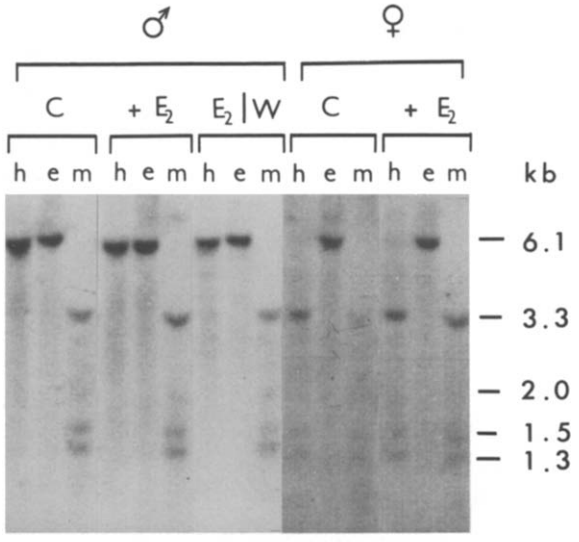

Fig.3. Absence of effects of oestradiol-17β on methylation pattern of the 3′-end of vitellogenin gene B1 in mature male and female *Xenopus*. DNA was extracted from parenchymal cells of mature male and female frogs 3 days after a single injection of oestradiol (E_2). To test a possible late effect of the hormone, cells were prepared from one group of male *Xenopus* 40 days after hormone administration (E_2/W). All other procedures and abbreviations as in fig.2. The panels showing DNA from control animals (C) are the same as panels for liver DNA (L) in fig.2.

after oestrogen administration. Instead of a single injection of oestradiol which produces a transient burst of transcription of vitellogenin genes, dying away by about 12 days, implantation of a cholesterol-based hormone pellet produces a sus-

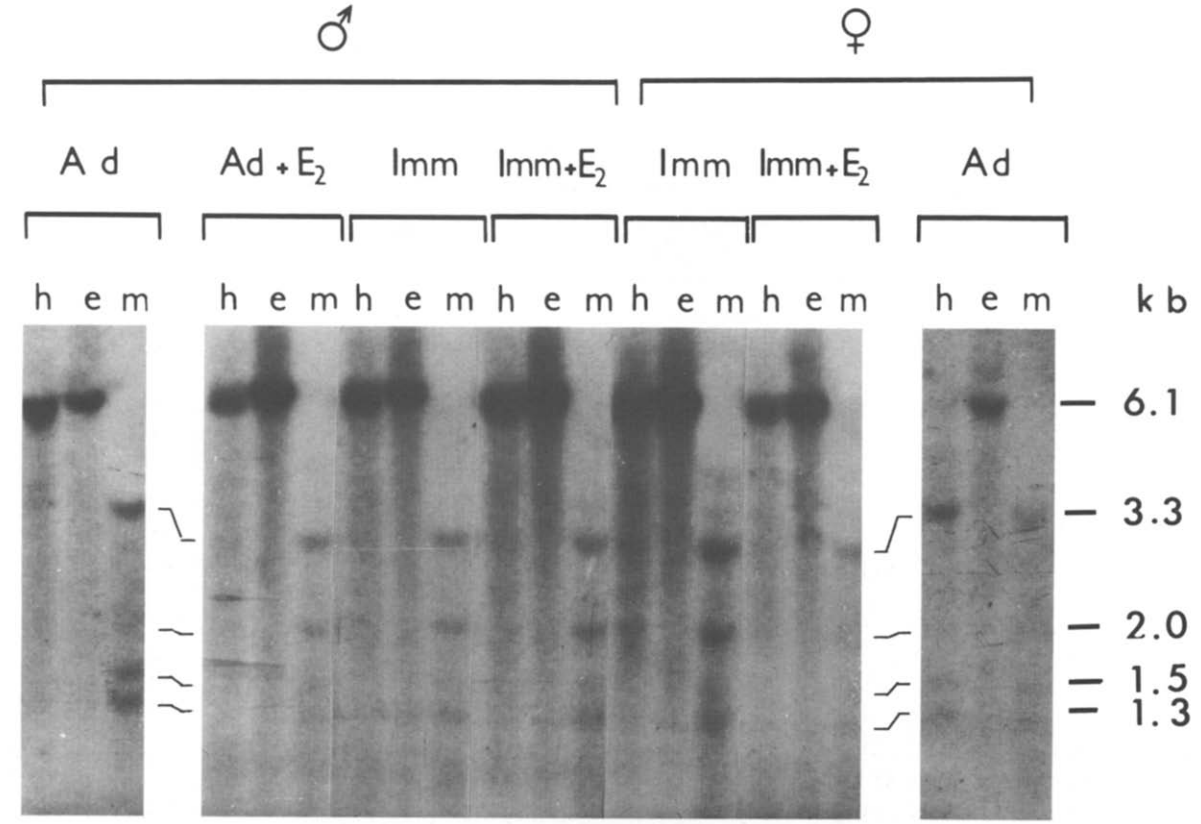

Fig.4. Differences and similarity in methylation patterns of liver DNA from immature and adult *Xenopus* of both sexes, with and without continuous exposure to oestrogen. DNA was extracted from parenchymal cells of adult male *Xenopus* implanted with a pellet of oestradiol 19 days earlier. Immature males and females were implanted with a pellet of 2–3 mg oestradiol for 7 days before the DNA was extracted. Ad, adult (>4 years); Imm, immature (9 months). All other procedures and abbreviations as in fig.2. The panels for adult male and female are the same as those depicting liver DNA (L) in fig.2.

tained transcription at maximum rates over several weeks in both males and females [15]. By switching to pellet implantation, the continuously actively transcribed vitellogenin genes B1 in male hepatocytes still remained fully methylated at the 3′-sites (fig.4). Such long-term oestrogen administration also did not alter the methylated state of these sites in tissues in which vitellogenin is not synthesized, such as oviduct, erythrocytes and kidney (not shown). Thus, the sex- and tissue-specific demethylation of gene B1 is insensitive to its physiological inducer or transcriptional status.

When, during development, do the 3′-sites of gene B1 become specifically demethylated in female *Xenopus* liver? In liver DNA from different developmental stages the 3′-end of gene B1 remained fully methylated in tadpole and froglets up to 4 months after fertilisation. However, in immature females at 9 months of development a low level of demethylation could be detected in liver DNA, relative to >4-year-old adults (fig.4). Quantitative densitometric scanning of the autoradiograms revealed that the extent of demethylation at 9 months of age had reached about 20% of the adult level (fig.5). Administration of oestrogen to these immature females did not enhance this level of demethylation nor induce the process in males, although the genes were hormonally sensitive and transcriptionally competent in both sexes at this stage. Thus the 3′-demethylation of vitellogenin gene B1 in female *Xenopus* liver parenchymal cells appears to be a gradual, rather than an abrupt, process during maturation.

The present finding of a sex- and tissue-specific demethylation at the 3′-end of a developmentally regulated gene is unexpected and novel in the light of the general consensus of opinion concerning the association between methylation of CCGG residues and transcriptional activity [1–3]. Taken together, our findings do not support the view that methylation and demethylation are a consequence of prolonged gene activity or inactivity [12,20,22], nor that a gene has to be continuously expressed to retain its undermethylated status [22]. The gradual demethylation observed during development also raises the question of whether the phenomenon reflects the developmental acquisition of vitellogenic competence of female *Xenopus* liver. In this context, it is important to note that the sexual differentiation of the liver is particularly

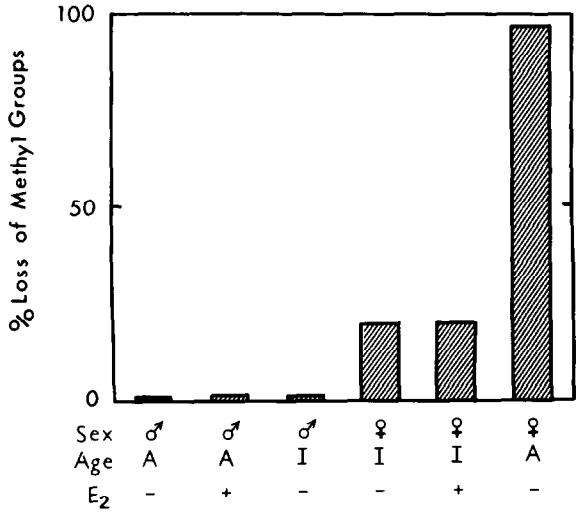

Fig.5. Quantitation of loss of methyl groups from the 6.1 kb 3′-region of vitellogenin gene B1 in liver parenchymal cells of adult and immature *Xenopus*. The autoradiogram shown in fig.4 was densitometrically scanned in a Joyce-Loebl Chromoscan and the results are expressed as the reduction in the fraction of signal due to the 6.1 kb fragment generated by *Eco*RI/*Hpa*II double digestion relative to restriction with *Eco*RI alone. A, adult; I, immature.

susceptible to pituitary and gonadal hormones during early development [23–25]. More generally, there is also the question of whether the ontogenic undermethylation at 3′-end is a peculiarity of *Xenopus* vitellogenin genes or is the phenomenon applicable to other developmentally regulated genes. It is relevant to consider recent findings of enhancer sequences which may developmentally activate genes at sites remote from the usual regulatory elements at the 5′-end [26,27]. In any event, the demethylation at the 3′-end may underlie a terminal determination of the potential for physiological expression of vitellogenin genes in a sex- and tissue-specific manner.

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